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| <p>(54) Title: A 987P FIMBRIA-E-PRODUCING MICROORGANISM, A VACCINE FOR THE IMMUNIZATION OF PIGS AS WELL AS A METHOD FOR PRODUCTION OF THE VACCINE</p>  |  |  |  |
| <p>(57) Abstract</p> <p>A 987P fimbriae-producing microorganism shows 987P fimbriae in an amount corresponding to five percent or more by weight of its protein content. For example, 10 percent by weight of purified 987P fimbriae can be extracted from <i>E. coli</i> NCTC 11,665 calculated from the total protein content of the cells. The microorganism is used in the form of whole cells and/or cell wall material for production of a vaccine for the immunization of pigs, for example, sows or sucking pigs, against pathogenic <i>E. coli</i> strains. The vaccine is produced by culturing of the 987P fimbriae-producing microorganism. If desired, the microorganism is then killed, and the cell wall material comprising 987P fimbriae is isolated whereupon it is mixed with a carrier substance and/or an adjuvant.</p> |  |  |  |

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-1-

A 987P fimbriae-producing microorganism, a vaccine for the immunization of pigs as well as a method for production of the vaccine.

The present invention relates to a 987P fimbriae-producing microorganism.

Pigs, especially sucking pigs, often suffer from diarrhoea caused by enteropathogenic coli bacteria. Even in herds where the pig breeder aims at a healthy environment for the pigs it is common for the breeder 5 to experience a considerable loss of sucking pigs on account of diarrhoea. It is, for example, common for the pigs of the so-called SPF status to suffer from diarrhoea caused by *E. coli* strains.

The pathogenic *coli* strains are characteristic by producing toxins and also by carrying type K88, K 99 or 987P fimbriae which fix the bacterium 10 to receptors on the epithelium of the intestine. These fimbriae are thread-like protein structures which consist of amino acid chains with a molecular weight of between 16,000 and 28,000 Dalton.

The enteropathogenic *coli* strains lose their pathogenic characteristics when they no longer express their adherence antigen, also even if they 15 still produce toxins. *E. coli* diarrhoea in pigs can, therefore, be avoided by preventing the bacteria from adhering to the intestinal wall. Administration of antibodies against K88 fimbriae counteracts e.g. colonization of K88 fimbriae-producing strains on the intestinal wall, and the enteropathogenic *coli* bacteria can, therefore, no longer exercise 20 their toxic effect in the intestinal tract.

987P fimbriae-producing *coli* bacteria have not been examined to the same extent as K88 and K99 fimbriae-producing bacteria, but in vitro experiments have shown that the adherence of 987P fimbriae-producing *coli* bacteria to the intestinal epithelium of pigs can be impeded by purified 25 Fab-fragments of specific 987P antibodies, just as it has been proved that purified 987P fimbriae impede the adherence of the bacteria to the intestinal epithelium. It means that 987P fimbriae can serve for the immunization of pigs against pathogenic *E. coli* bacteria of serotype 987P.

R.E. Isaacson and P. Richter have in *J. Bact.* 146, p. 784-789, (1981) 30 described the production of purified 987P fimbriae which may be used in such a vaccine for the immunization of pigs against pathogenic *coli* bacteria of serotype 987P, but the resultant production of fimbriae is low so that the vaccine becomes expensive.

-2-

It has now quite surprisingly been found that there are *E. coli* strains which produce 987P fimbriae in several times higher quantities than the strains known so far. It has thus become possible to produce the above-mentioned vaccine against pathogenic *coli* bacteria in a considerably less cost-consuming way. Besides, the *E. coli* strains dealt with can in some cases be used directly in the crude form as vaccine because as compared to the amount of fimbriae they contain many times smaller amounts of toxins.

Accordingly, a 987P fimbriae-producing microorganism has been obtained according to the invention, which microorganism has 987P fimbriae in an amount corresponding to five percent or more by weight of its protein content.

Different protein determination methods can be used for determination of the 987P fimbriae-production of a microorganism, e.g. the Lowry method (see *J. Biol. Chem.*, 193, 265, (1951)) or electrophoresis, e.g. SDS-page electrophoresis.

According to the invention the microorganism may be a 987P-producing *E. coli* bacterium. It may be a natural toxin-producing *coli* bacterium or an artificially produced *coli* bacterium from which the toxin-producing characteristic has been removed or weakened, e.g. by genetic engineering.

The bacterium may also be a naturally non-toxic *coli* bacterium whose chromosome has been replaced partially, e.g. by a genetic engineering technique, by parts of the chromosome from the 987P fimbriae-producing *E. coli* bacterium in question. Chiefly, only the gene responsible for the 987P fimbriae production has been transferred, but on the other hand not the gene which is responsible for the toxin production. Such a bacterium without toxin-producing characteristics has the advantage of being directly applicable as a vaccine, with no risk of harming the vaccinated animal.

It is possible through genetic engineering to transfer genes from a chromosome to plasmids which in turn are transferred to a non-toxic cell. The organism according to the invention may, therefore, also be a non-toxic *E. coli* bacterium which contains a plasmid with the above mentioned gene for production of 987P fimbriae.

-3-

The microorganism according to the invention produces 987P fimbriae e.g. in an amount corresponding to 5-15 percent by weight of its protein content. By experiment an E. coli strains has thus been produced which produces 987P fimbriae in an amount corresponding to approx. 10 percent by weight of its protein content. This strain is deposited with National Collection of Type Cultures (NCTC), England, under No. 11,665.

Accordingly, the microorganism according to the invention in the chromosome may contain the entire gene or a fragment of the gene responsible for the fimbria production of the E. coli strain deposited under No. 11,665 in the NCTC.

The organism according to the invention may thus be the deposited strain itself or an organism which through genetic engineering has obtained its 987P fimbriae-producing characteristic from the said strain.

The gene for 987P fimbria production of the said strain can also be 15 transferred to a plasmid of another organism through genetic engineering.

Accordingly, the microorganism according to the invention may contain a plasmid with a fragment responsible for the fimbria production of an E. coli strain deposited under No. 11,665 in the NCTC.

The microorganism according to the invention can be used for production 20 of a vaccine for the immunization of pigs against pathogenic 987P fimbriae-carrying E. coli bacteria.

Accordingly, the invention also relates to a vaccine for the immunization of pigs. The vaccine according to the invention is characteristic in that it contains whole cells and/or cell wall material of a microorganism which 25 produces 987P fimbriae in an amount corresponding to five percent or more by weight of its protein content together with a carrier substance and/or an adjuvant.

The vaccine may thus contain living or dead whole cells of the micro-organism. If the organism has no toxin-producing gene the vaccine can be 30 administered without hesitation. If on the other hand the organism is toxin-producing, like the above-mentioned E. coli strain NCTC No. 11,665, the vaccine should be administered with caution in order to avoid disease-causing attacks of diarrhoea.

-4-

As a rule, sows will be able to resist a toxin-containing vaccine and transfer the antibodies formed to their sucking pigs via the colostrum.

On the other hand, sucking pigs will not be able to resist a toxin-containing vaccine. In this case a vaccine of non-toxic whole cells can be used, or a vaccine of non-toxic parts of the cell wall material from toxic cells, e.g. the purified fimbriae of the cells.

Ordinary feed and aqueous solutions can be used as carrier substances, e.g. a physiological NaCl solution.

Aluminium hydroxide such as "Alhydrogel" from Superfos can be used as 10 an adjuvant.

The vaccine according to the invention can be injected parenterally, e.g. subcutaneously or intramuscularly, into the animal so that antibodies are formed which prevent natural toxic *E. coli* bacteria from adhering to the intestinal epithelium where they might otherwise give off diarrhoea- 15 causing toxins.

A vaccine of living cells can be administered orally which is an economic advantage e.g. with sucking pigs. The fimbriae of the vaccine will occupy the receptors of the intestinal wall, which will not be accessible as a place of adherence for natural toxic *E. coli* bacteria.

20 Oral administration of the vaccine may also cause formation of antibodies in the intestinal wall which will amplify the protective effect which is obtained by occupation of the intestinal receptors for 987P fimbriae.

Oral or parenteral administration into sows will cause formation of antibodies which via the sow's colostrum are transferred to the sucking pigs 25 which are protected against coli diarrhoea. In order to produce the said formation of antibodies 2.5-5 mg of fimbria protein can be injected intramuscularly into sows six and three weeks before farrowing. In the case of re-vaccination it will only be necessary to vaccinate the sows three weeks before farrowing.

30 A microorganism in the form of a 987P fimbriae-producing *E. coli* bacterium can be used for the vaccine. Chiefly an organism is used which has 987P fimbriae in an amount corresponding to 5-15 percent by weight of its protein content.

-5-

The vaccine according to the invention can be produced on the basis of a microorganism which in the chromosome contains all the genes or a fragment of the gene responsible for the fimbria production of an E. coli strain deposited under No. 11,665 in NCTC.

5 The vaccine according to the invention can also be produced on the basis of a microorganism which contains a plasmid with a fragment of the gene responsible for the fimbria production of an E. coli strain deposited under No. 11,665 in NCTC.

The vaccine according to the invention can also be made on the basis of 10 a the E. coli bacterium NCTC No. 11,665 itself or an organism which has been given the 987P fimbriae-producing characteristics by genetic engineering.

The invention also relates to a method for production of the above-mentioned vaccine, and this method is characteristic in that a microorganism 15 which produces 987P fimbriae in an amount corresponding to five percent by weight of its protein content is cultured. If desired, the microorganism is then killed and the cell wall material comprising 987P fimbriae is isolated whereupon it is mixed with a carrier substance and/or an adjuvant.

20 The invention is explained in greater detail in connection with the following examples.

-6-

Example 1

Isolation of Fim<sup>+</sup> colonies

A test tube with 10 ml BHI (Brain Heart Infusion Broth, Difco) is seeded with an *E. coli* strain, serotype O9: K103: 987P, received from the State Serum Institute, Copenhagen. Culturing is carried out for 14 days at 37°C without shaking.

If a bacterium is fimbriated, it fills more relative to its weight, and fimbriated bacteria cells will, therefore, have a tendency to seek towards the surface during culturing without shaking.

10 Accordingly, samples are only drawn from the surface layer of the seeded tube. They are spread on blood agar plates consisting of BHI with 2% agar and 5% red blood cells from horses and cultured for 16 hours at 37°C. The white colonies are tested with an antiserum which agglutinates with 987P fimbriae (serotype O20:987P). Few colonies show agglutination during this first culturing.

A test tube with 10 ml BHI is seeded with samples from the surface layer in the tubes which show the strongest agglutination and they are cultured for five to eight days without shaking at 37°C.

20 Samples from the surface layer in the test tubes are seeded into new test tubes with 10 ml BHI and cultured without shaking at 37°C. After two to three days pellicles appear on the surface in some of the tubes. Samples of the pellicles are spread on blood agar plates, and the white colonies are tested for agglutination. Considerably more agglutinating colonies are now obtained.

25 After repeated re-seedings of agglutinating colonies and of pellicle layers in BHI and culturing at 37°C for two to three days without shaking, cultures are obtained with an increasingly higher frequency of bacteria in the Fim<sup>+</sup> phase.

After approx. 1 1/2-2 months colonies are isolated which after re-seeding on a fresh blood agar plate every day remain in the Fim<sup>+</sup> phase during the whole of the subsequent test period of approx. three months.

-7-

Obtainment of NCTC 11,665

Culturing of the  $\text{Fim}^+$ -stable colonies is continued in BHI under the same conditions as described above, and the pellicle which has formed is spread and cultured on blood agar plates.

5 In order to examine the fimbria production of the colonies the cells are boiled in a buffer containing 3% SDS (Sodium dodecylsulphate) and 1% 2-mercaptoethanol, and the protein material obtained is examined by electrophoresis. A drop of the material is mixed with a drop of a 987P fimbria preparation on an SDS polyacrylamide-gel and developed with 10 Coomassie Brilliant Blue G (see Example 2 for further details). On the basis of the 987P fimbria band (23,000 Dalton) it is possible to make an estimate of the fimbria production of the cells. There are none or only weakly coloured bands corresponding to a low fimbria production of the colonies.

15 It is tried to separate coloured bands further using Western blotting which confirms that the protein in the band is 987P fimbriae.

After many re-seedings without major changes, a colony appears unexpectedly which gives a strongly coloured band by electrophoresis. It is a special coli strain which produced 987P fimbriae in an exceptionally high amount as the fimbria protein constitutes a much higher proportion of the total protein content of the cell, evaluated from the degree of colouring of the protein bands.

The strongly fimbriae-producing coli strain was deposited with the NCTC under No. 11,665.

25

Example 2

Culturing

1 litre of BHI bouillon is seeded with the NCTC 11,665 strain, and incubation takes place at 37°C for approx. 16 hours during shaking.

Extraction of 987P Fimbriae

30 The 987P fimbriae-carrying cells formed during culturing are extracted, and the fimbriae are purified according to the method described by Issacson and Richter in J. Bact. 146, p. 874-789 (1981).

-8-

The culture medium is centrifuged at 10,000 rev./min. for approx. 10 minutes at 4°C, and the precipitate is suspended in 100 ml of 0.01 M MOPS (3-N-morpholino) propane-sulphonic acid), (pH 7.2). The suspension is homogenised in an Omni mixer for approx. 10 minutes, and the homogenised suspension is centrifuged at 10,000 rev./min. for 10 minutes. The supernatant is decanted and its pH is adjusted to 3.9 using 1 N acetic acid. After stirring for half an hour the fimbriae are precipitated, and the suspension is centrifuged for 20 minutes at 12,000 rev./min. The precipitate is dissolved in 50 ml MOPS during stirring on 10 an ice bath for at least three hours, stands for 16 hours at 4°C, and is stirred on an ice bath for one hour. The solution is centrifuged at 12,000 rev./min. for 20 minutes to remove undissolved material.

An equal volume of a crystallization buffer is added to the supernatant. The buffer consists of 0.09 M MOPS (pH 7.2), 0.2 M MgCl<sub>2</sub> and 1.7% NaCl. 15 During stirring on an ice bath for half an hour, the 987P fimbriae are precipitated and collected by centrifugation at 10,000 rev./min. for 10 minutes.

The precipitate is re-suspended during stirring in MOPS, and the suspension is treated in the same way as described above. The solution obtained is centrifuged at 12,000 rev./min. for 20 minutes at 4°C. The supernatant contains the 987P fimbriae in a dissolved state.

The above described procedure consisting of addition of a crystallization buffer to the supernatant, collection of precipitated material by centrifugation, dissolution of the precipitate in MOPS, and centrifugation of 25 the solution for obtainment of a supernatant solution of 987P fimbriae is repeated at least four times.

The last crystallization results in a pure white precipitate of 987P fimbriae which after freeze-drying weighs 250 mg corresponding to approx. 10% of the total protein content of the collected cells.

3

Purity and yield

The purity and yield of the individual stages are determined by SDS-page electrophoresis according to Laemmli, *Nature*, 227, 680-685, (1970). For the electrophoresis a 15% low bis-polyacrylamide-gel is used which consists of 11.9 mg H<sub>2</sub>O, 15 ml 40% acrylamide, 2 ml 1.5% bis-acrylamide, 400 ul 10% SDS, 400 ul 10% glycerol, 300 ul 10% ammonium persulphate solution, and 20 ul TEMED in 10 ml 1.5 M Tris-H<sub>2</sub>O, (pH 8.8). A 6% stacking gel is used which consists of 9.5 ml H<sub>2</sub>O, 3 ml 40% acrylamide, 2.1 ml 1.5% bis-acrylamide, 200 ul 10% SDS, 100 ul 10% glycerol, 150 ul ammonium persulphate and 20 ul TEMED in 5 ml 0.5 M Tris-HCl (pH 6.8).

The sample is boiled for two minutes in a buffer containing 3% SDS and 1% 2-mercaptoethanol and transferred to the gel. 25 mM Tris-HCl and 250 mM glycine in a 1% SDS solution are used as electrophoresis buffer. The electrophoresis is carried out at 85 V for 14 hrs (corresponding to approx. 15 1200 volt hours). The gel is developed by Coomassie Brilliant Blue G.

At a molecular weight of 23,000 Dalton there are very strong bands of 987P fimbriae in both culture medium, intermediate products and final products, compared to the bands from the previously isolated 987P fimbriae-producing *E. coli* bacteria.

20 In a similar way, yield and purity are followed using Western blotting. The protein material is fractionated by means of the above-mentioned SDS-page electrophoresis, but instead of the 15% low bis-polyacrylamide-gel a 15% high bis-polyacrylamide-gel is used. The fractionated protein substances are blotted onto nitrocellulose paper at a voltage of 25 V for 16 hours. The paper or filter is washed in water for approx. one minute, in Tween buffer (10 mM Tris-HCl, 155 mM NaCl in 0.5% Tween 20 (pH 7.4)) for 30 minutes, and again in water for one minute. The filter is placed in a large glass tube, and rabbit antiserum (09:K103:987P) dissolved in Tween buffer is added. The tube is closed and rotated for 60 minutes. The filter is washed in water for one minute, in Tween buffer for half an hour, again in Tween buffer for half an hour, and then in water for one minute.

-10-

The filter is incubated in a glass tube with protein A conjugated with peroxidase (from Sigma Laboratories) and washed twice with Tween buffer for half an hour. The filter is placed on a glass plate for development of colour and is then covered with a freshly made substrate solution (80 5 mg dioctylsodium sulphosuccinate (DONS) and 24 mg tetramethylbenzidine (TMB) dissolved in 10 ml ethanol added to 30 ml mM citric acid and 20 mM  $\text{Na}_2\text{HPO}_4$  (20  $\mu\text{l}$  40%  $\text{H}_2\text{O}_2$  is added just before use). The colour development is stopped with a stop buffer.

10 Strong colourings are again seen opposite to the band corresponding to the 987P fimbriae protein, and the purity of the 987P fimbria product proves to be high since there are no other bands with the homologous antiserum used.

The purity of the product is confirmed by determination of the amino acid 15 composition as described by Klemm in Eur. J. Biochem., 117, p. 617-627, (1981).

The product proves to be more than 98% pure which is in accordance with the SDS-page electrophoresis. The analysis for amino acids is in accordance with the fact that the N-terminal amino acid in 987P fimbriae is 20 alanine (Isaacson and Richter, 1981).

#### Amino acid sequencing

Manual Edman degradation was carried out on the obtained 987P protein as described by Klemm in the J. Biochem., 117, p. 617-627 (1981). The following sequence was found: Ala - Pro - Ala - Glu - Asn - Asn - Thr - Ser - 25 Glx - Ala - Asx - Leu.

#### Example 3

#### Production of oral test vaccine

A culture of the NCTC 11, 665 bacterium is spread on a blood agar plate and cultured at 37°C for 16 hours. White colonies are seeded into 10 ml BHI 30 and incubated at 37°C for 16 hours. 0.1 ml of the culture medium obtained is inoculated into 200 ml UHT milk which is kept at 37°C without shaking for 16 hours.

-11-

Production of intramuscular test vaccine

150 mg of the fimbria product obtained in Example 3 is suspended in a solution consisting of 240 ml 0.9% NaCl solution and 60 ml Alhydrogel.

Production of a challenge bacterial culture

5 10 ml BHI is inoculated with three different heterologous toxic bacterial strains, all of serotype 987P. Culturing is carried out for 16 hours at 37°C during shaking. The culture medium obtained is poured into 100 ml BHI, and cultured for 16 hours at 37°C during shaking.

Vaccination test

10 A total of 28 pregnant sows are used for the test. A control group of eight sows is given a normal feed. Another group of sows is given a feed into which 200 ml of the oral test vaccine is mixed on three subsequent days, at the latest 10 days before farrowing.

15 A third group of sows is given a normal feed, but they have 5 ml of the intramuscular vaccine injected six and three weeks before farrowing. A fourth group of sows is also given normal feed, but they receive the recommended dose of 2 ml of another 987P fimbriae-containing vaccine intramuscularly six and three weeks before farrowing.

20 The udders of all the sows are swabbed morning and evening with the challenge bacterial culture from one day before farrowing till one day after farrowing.

The faeces of the sucking pigs are examined one day after farrowing by means of a swab test and evaluated according to the following scale:

25            0    = normal firm to semi-soft faeces  
              +    = soft, mucous faeces  
              ++   = soft to watery faeces  
              +++ = profuse watery diarrhoea, dehydration

30 It can be seen from the results in Tables 1 and 2 that the vaccine according to the invention administered to sows before farrowing reduces the occurrence of diarrhoea and the mortality of the sucking pigs exposed to toxic 987P E. coli bacteria.

It also appears from Tables 1 and 2 that the vaccine according to the invention is more effective against diarrhoea than the commercial vaccine.

-12-

Table 1

| Experim.<br>series  | Sow<br>No.   | Live-<br>born                            | Still-<br>born                       | Squeezed<br>to death                 | Faeces<br>++                         | Faeces<br>+++                          | Dead suck.<br>pigs                   |
|---|--|--|--------------------------------------|--------------------------------------|--------------------------------------|--|--------------------------------------|
| 1) Con-<br>trol-<br>group                                       | 2113<br>2047<br>2149<br>2158<br>1997<br>2157<br>1860<br>2300 | 11<br>6<br>6<br>11<br>12<br>9<br>4<br>11 | 0<br>0<br>0<br>0<br>0<br>0<br>0<br>0 | 2<br>0<br>0<br>0<br>0<br>1<br>0<br>0 | 6<br>0<br>0<br>0<br>0<br>0<br>0<br>1 | 0<br>6<br>6<br>11<br>12<br>8<br>4<br>8 | 0<br>0<br>5<br>7<br>4<br>0<br>0<br>1 |
|   | Total  | 70                                       | 0                                    | 3                                    | 7                                    | 55                                     | 17                                   |
| 2) Oral<br>vaccine<br>acc. to<br>invent-<br>ion                 | 2108<br>2050<br>2049<br>2093<br>2000<br>2360                 | 9<br>5<br>11<br>3<br>9<br>7              | 0<br>0<br>0<br>6<br>0<br>0           | 0<br>0<br>0<br>0<br>0<br>0           | 0<br>0<br>0<br>0<br>0<br>0           | 5<br>3<br>0<br>0<br>0<br>0             | 0<br>0<br>1<br>0<br>0<br>0           |
|   | Total  | 44                                       | 6                                    | 0                                    | 0                                    | 8                                      | 1                                    |
| 3) Intra-<br>muscu-<br>lar<br>vacc.<br>acc.to<br>invent-<br>ion | 2098<br>2032<br>2302<br>2299<br>2350<br>2231<br>2216         | 9<br>8<br>9<br>8<br>8<br>8<br>10         | 0<br>0<br>0<br>0<br>0<br>0<br>0      | 0<br>0<br>0<br>0<br>0<br>0<br>0      | 2<br>1<br>1<br>2<br>4<br>1<br>1      | 0<br>0<br>0<br>0<br>0<br>0<br>1        | 0<br>0<br>0<br>1<br>0<br>0<br>0      |
|   | Total  | 60                                       | 0                                    | 0                                    | 12                                   | 1                                      | 1                                    |
| 4) Intra-<br>muscu-<br>lar<br>commer-<br>cial<br>vaccine        | 2111<br>2072<br>2232<br>2295<br>2325<br>2342<br>2349         | 11<br>10<br>11<br>10<br>4<br>6<br>4      | 0<br>0<br>0<br>0<br>0<br>0<br>0      | 0<br>0<br>0<br>0<br>1<br>0<br>1      | 5<br>3<br>2<br>1<br>0<br>2<br>0      | 3<br>6<br>2<br>4<br>0<br>2<br>0        | 0<br>0<br>0<br>0<br>0<br>0<br>0      |
|   | Total  | 56                                       | 0                                    | 2                                    | 13                                   | 17                                     | 0                                    |

-13-

Table 2

| Experimental series                                   | Diarrhoea attacks<br>(++ and +++) | Mortality<br>(dead sucking pigs) |
|---|-----------------------------------|----------------------------------|
| 1) Control group                                      | 88.6%                             | 24.9%                            |
| 2) Oral vaccine acc.<br>to invention                  | 18.2%                             | 2.3%                             |
| 3) Intramuscular<br>vaccine according<br>to invention | 21.7%                             | 1.7%                             |
| 4) Intramuscular<br>commercial vaccine                | 53.6%                             | 0.0%                             |

-14-

International Application No: PCT/ D K86/00012

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 3 line 5-6 of the description <sup>1</sup>A. IDENTIFICATION OF DEPOSIT <sup>2</sup>Further deposits are identified on an additional sheet  <sup>3</sup>Name of depositary institution <sup>4</sup>

National Collection of Type Cultures

Address of depositary institution (including postal code and country) <sup>4</sup>Central Public Health Laboratory  
61 Colindale Avenue  
London NW9 5HT, EnglandDate of deposit <sup>5</sup>

1st june 1984

Accession Number <sup>6</sup>

11.665

B. ADDITIONAL INDICATIONS <sup>7</sup> (leave blank if not applicable). This information is continued on a separate attached sheet C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE <sup>8</sup> (If the indications are not for all designated States)D. SEPARATE FURNISHING OF INDICATIONS <sup>9</sup> (leave blank if not applicable)The indications listed below will be submitted to the International Bureau later <sup>10</sup> (Specify the general nature of the indications e.g., "Accession Number of Deposit")E.  This sheet was received with the international application when filed (to be checked by the receiving Office)

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- 15 -

Claims

1. A 987P fimbriae-producing microorganism, characterized by 987P fimbriae in an amount corresponding to five percent or more by weight of its protein content.
- 5 2. A microorganism as claimed in claim 1, characterized in that it is a 987P fimbriae-producing *E. coli* bacterium.
3. A microorganism as claimed in claims 2-3, characterized by 987P fimbriae in an amount corresponding to 5-15 percent by weight of its protein content.
- 10 4. A microorganism as claimed in claims 1-3, characterized in that it contains in the chromosome the entire gene or a fragment of the gene responsible for the fimbria production of an *E. coli* strain deposited under No. 11,665 in the NCTC.
5. A microorganism as claimed in claims 1-4, characterized in 15 that it contains a plasmid with a fragment of the gene responsible for the fimbria production of an *E. coli* strain deposited under No. 11,665 in the NCTC.
6. A vaccine for the immunization of pigs, characterized in that it contains whole cells and/or cell wall material of a microorganism 20 which produces 987P fimbriae in an amount corresponding to five percent or more by weight of its protein content, together with a carrier substance and/or an adjuvant.
7. A vaccine as claimed in claim 6, characterized in that the microorganism is a 987P fimbriae-producing *E. coli* bacterium.
- 25 8. A vaccine as claimed in claims 6-7, characterized in that the microorganism has 987P fimbriae in an amount corresponding to 5-15 percent by weight of its protein content.
9. A vaccine as claimed in claims 6-8, characterized in that the microorganism in the chromosome contains the entire gene or a fragment 30 of the gene responsible for the fimbria production of an *E. coli* strain deposited under No. 11,665 in the NCTC.

- 16 -

10. A vaccine as claimed in claims 6-9, characterized in that the microorganism contains a plasmid with a fragment of the gene responsible for the fimbria production of an *E. coli* strain, deposited under No. 11,665 in the NCTC.
11. A method for production of the vaccine as claimed in claim 6, characterized in that a microorganism is cultured which produces 987P fimbria in an amount corresponding to five percent or more by weight of its protein content and if desired, the microorganism is killed and the cell wall material comprising 987P fimbriae is isolated, whereupon it is mixed with a carrier substance and/or an adjuvant.
12. A method as claimed in claim 11, characterized in that a 987P fimbriae-producing *E. coli* bacterium is used.
13. A method as claimed in claims 11-12, characterized in that an organism is used having 987P fimbriae in an amount corresponding 15 to 5-15 percent by weight of its protein content.
14. A method as claimed in claims 11-13, characterized in that an organism is used containing in the chromosome the entire gene or a fragment of the gene responsible for the fimbria production of an *E. coli* strain deposited under No. 11,665 in the NCTC.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/DK86/00012

## I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC 4

C 12 N 1/20, A 61 K 39/02 // C 12 R 1:19

## II. FIELDS SEARCHED

Minimum Documentation Searched ?

| Classification System | Classification Symbols  |
|-----------------------|---|
| IPC 4<br>US Cl        | C 12 N 1/20; C 12 N 15/00; A 61 K 39/02, /40, /108<br>424:87, 88, 92; 435:253 |

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched \*

SE, NO, DK, FI classes as above

## III. DOCUMENTS CONSIDERED TO BE RELEVANT\*

| Category * | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>  | Relevant to Claim No. 13 |
|------------|---|--------------------------|
| X, Y       | Infection and Immunity Vol 22, Nr 3, December 1978, Morgan et al. "Immunization of Suckling Pigs against Enterotoxigenic Escherichia coli - Induces Diarrheal Disease". p 771-777 | 1-14                     |
| X          | Infection and Immunity, August 1980, Vol 29, Nr 2, Isaacson et al. "Immunization of Suckling Pigs...". p 824-826  | 1-14                     |
| X          | Patent abstracts of Japan, abstract of JP 59-20226 published 1984-02-01   | 1-14                     |
| X          | US, A, 4 237 115 (CHARLES C BRINTON JR., PITTSBURGH PA.)<br>2 December 1980<br>& US, 4454116  | 1-14                     |
| X          | Journal of Bacteriology, May 1981, Isaacson et al. "Escherichia coli 987P Pilus: Purification and Partial Characterization". p 784-789  | 1-5<br>.../...           |

\* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

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"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

1986-04-21

1986-05-05

International Searching Authority

Signature of Authorized Officer

Swedish Patent Office

*Yvonne Siösteen*  
Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1986)

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## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category | Citation of Document, with indication, where appropriate, of the relevant passages                                    | Relevant to Claim No |
|----------|---|----------------------|
| Y        | EP, A3, 0 107 845 (BACTEX INCORPORATED,<br>PITTSBURGH, PA 15213 (US),<br>1984-05-09<br>& AU, 20662/83<br>JP, 59095222 | 1-14                 |
| X        | EP, A3, 0 060 129 (CETUS CORPORATION BERKELY,<br>CALIFORNIA (US)<br>1982-09-15  | 1-14                 |